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(57) Abstract

A method and kit for the extraction of DNA from plants is provided, which quickly yields plant DNA with a high level of purity. The method isolates DNA (genomic, chloroplast, and/or mitochondrial DNA) using immobilized anionic groups, preferably on a chromatographic substrate or more preferably magnetic beads derivatized with anionic groups such as diethylaminoethyl (DEAE) via an anion–exchange interaction. The purified DNA is then eluted with ions (typically a salt solution). RNA can be removed by digestion with RNAse.

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MAGNETIC DNA EXTRACTION KIT FOR PLANTS

Background of the Invention

The present invention generally relates to the field of methods and kits for the extraction of DNA and specifically to methods and kits for the extraction of DNA from plants.

Methods for extracting DNA from plants include:

The Dellaporta method. Dellaporta, S.L., et al. 1983. A plant DNA minipreparation: Version II. *Plan Mol Biol Rep* 1:19-21, where DNA is precipitated to produce a very crude preparation, with a lot of the ethanol insoluble contaminants present in the solution. A variant of the Dellaporta method introduces an additional step of purifying the DNA via ultracentrifugation through a cesium chloride (CsCl) gradient for several hours or overnight. Richards, E., et al. 1994. *Current Protocols in Molecular Biology*, Suppl. 27, 2.3.1-2.3.7. Purifying DNA through CsCl gradients is an old established technique that can give very pure DNA, but is time consuming, expensive and results in very low yields.

An alternative DNA extraction method is the cetyltrimethylammonium bromide (CTAB) method. Murry, M.G. et al. 1980. *Nucleic Acids Res*, 8:4321-4325. CTAB is a cationic detergent, which will form an insoluble complex with the DNA in the presence of low concentrations of salt, such as a 0.5 M sodium chloride (NaCl) solution. In the CTAB method, the original lysis solution contains about 1.4 M NaCl. The DNA binds with the CTAB when the NaCl concentration is decreased to 0.5 M. This method is also time consuming and, because the procedure does not use organic solvents, an additional step needs to be included to clean up the DNA: a final extraction with organic solvents to rid the preparation of polysaccharide and phenolic compounds.

Methods for extraction of DNA from bacterial cells are more well established. Usually cells are lysed by placing the cells in an alkaline environment. Plasmid DNA is separated from bacterial chromosomal DNA by precipitating the chromosomal DNA and leaving the plasmid DNA in the

supernatant solution. As described in U.S. Patent No. 5,665,544 to Reeve, et al, after the chromosomal DNA is separated from the plasmid DNA, magnetic beads are introduced into the plasmid containing supernatant. The plasmid DNA binds to the beads, and the plasmid DNA is separated from the contaminates in the supernatant. U.S. Patent No. 4,935,342 to Seligson, et al., discloses a general method for the isolation and purification of nucleic acids, based on the binding to and elution from anion exchange columns, including the use of sequential columns containing weak and strong anion exchange materials. U.S. Patent No. 5,705,628 to Hawkins, discloses the use of magnetic microparticles with a coating including functional groups, specifically carboxyl or negatively charged groups, for the purification and isolation of DNA by binding and elution. International Application WO 96/18731 by Deggerdal, et al., discloses a method for the isolation of nucleic acids by the binding to and elution from a solid support, preferably magnetic beads, in the presence of a detergent, preferably an anionic detergent such as SDS or SARCOSYL™. U.S. Patent No. 5,650,506 to Woodard, et al., discloses the use of glass fiber membranes bearing positive surface charges for DNA purification by binding to and elution from the glass fibers.

A commercially available method for the extraction of DNA from bacterial cells is marketed as ISOLATE™ by Annovis, Inc. (catalog number 2-0300-85). The protocol includes the following steps: suspending bacterial cells in a buffer (pH 8.0), lysing the cells by mixing the suspension with an alkaline – detergent solution (0.2 M NaOH, 1% SDS), neutralizing the alkali and precipitating the chromosomal DNA with 3.0 M Potassium Acetate, pH 5.5, centrifuging the mixture to sediment the precipitated protein, cell debris and denatured chromosomal DNA, mixing the supernatant containing the plasmid DNA with a suspension containing magnetic beads, binding the DNA to the beads via an anionic interaction, eluting the DNA from the beads, and adjusting the salt concentration to 0.3, adding ethanol to render the plasmid DNA insoluble and effecting the precipitation of plasmid DNA from the liquid phase. This procedure does not work well with genomic plant DNA, however.

It is therefore an object of the present invention to provide a method of extracting and purifying DNA from plants with a high degree of purity and integrity in a short period of time.

It is also an object of the present invention to provide a kit with reagents for rapidly and inexpensively extracting and purifying DNA from plants.

Brief Summary of the Invention

A method and kit for the extraction of DNA from plants is provided, which quickly yields plant DNA with a high level of purity. The method isolates DNA (genomic, chloroplast, and/or mitochondrial DNA) using immobilized anionic groups, preferably on a chromatographic substrate or more preferably magnetic beads derivatized with anionic groups such as diethylaminoethyl (DEAE) via an anion-exchange interaction. The purified DNA is then eluted with ions (typically a salt solution). RNA can be removed by digestion with RNAse.

Detailed Description of the Invention

The following terms are used herein:

anionic - positively charged

cationic - negatively charged

nonionic – non charged

Zwitterionic – same number of positive and negative charges on opposite ends so total compound is neutral.

The method described herein can be used with any plant material and has been demonstrated to be efficacious with the following representative types of plants: arabdopsis seedlings, barley embryos, tobacco leaves, tomato leaves, soybean hypocotylis and cultured cells, white beans hypocotylis and roots, young and old pine needles.

The process generally includes the steps of: grinding plant material to make a tissue extract, lysing the plant cells, removing cell debris, and binding the plant DNA to an immobilized or insoluble material such as magnetic beads, where it is separated into pure form.

In a preferred method, plant material is first ground to a powder in liquid nitrogen and then incubated in lysis buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 0.25 M NaCl and 100 microgram/ml proteinase K) in the presence of a surfactant or nonionic detergent such as N-laurylsarcosine (SARKOSYL™), TRITON™ or NONIDET™ P-40, which acts to solubilize cell components and lyse the cells. Representative detergents are listed in Table I.

Cell debris is then removed by centrifugation and the supernatant, which contains the DNA and other soluble cell components, is collected. The DNA is then bound to an immobilized or insoluble material having anionic groups bound thereto, such as magnetic beads derivatized with DEAE. This material is mixed with the supernatant, bound to the DNA, and then removed from the supernatant using a magnetic separator.

The beads are subsequently washed and then the DNA is preferably eluted from the beads by adding NaCl to a final concentration of 1.0 M. Alternatively, the DNA could be removed by binding to DEAE chromatographic material or filler material, which is separated by washing, centrifugation, or other methods known to those skilled in the art.

The method and kit produces DNA of equal purity to CsCl gradient methods at yields that are equal to, or better than, the prior art, Dellaporta and CTAB methods. The specific problems of the Dellaporta method (use of a CsCl column), and CTAB method (use of cationic detergents), are avoided by this method. The method and kit also extracts and purifies the DNA more quickly than the CsCl method. The method including the precipitation step takes a total of approximately 2.5 hours; without the precipitation step it takes less than 2 hours (1 hour and 50 minutes) to extract and purify the plant DNA: approximately 1 hour to lyse the cells, 5 minutes to bind the DNA to the magnetic beads, 5 minutes to wash the beads, 5 minutes to separate the DNA from the beads, 30 minutes to precipitate the DNA from solution, and 40 minutes to collect the DNA by centrifugation. In contrast, the CsCl centrifugation requires many hours to purify the DNA.

The preferred method described herein produces a very pure DNA preparation via an anion-exchange interaction where DNA is replaced as the binding species on the anion-exchange matrix by chloride ions or other negatively charged ions derived from any of a variety of salts. If a traditional anion-exchange column were used at the point where the beads are introduced, the columns would likely clog, preventing collection of bound DNA, or if the elution was effected with strong acid or base, significant levels of contaminates would co-elute with the DNA. In contrast, since the DNA bound to the beads can be thoroughly mixed with the wash solutions, contaminates are more easily removed than they are in traditional column formats, resulting in a more pure preparation. Moreover, because the DNA bound to the beads is not sheared or compressed, when pelleted, the attached DNA consists of longer and more intact strands.

The lysis methods for plant genomic DNA and bacterial plasmid DNA are different. The lysis method for bacterial plasmid DNA uses alkaline conditions in the presence of anionic detergent in the form of SDS, while the lysis method for plant genomic DNA uses a nonionic detergent. The type of detergent used affects the remaining steps in each method. Since SDS is anionic (negatively charged, like DNA), it must be removed from the solution in the plasmid procedure before the beads are introduced, otherwise the SDS would compete for binding with the plasmid DNA on the positively charged beads. In the ISOLATE™ plasmid extraction system, the SDS is removed from the solution by adding potassium acetate to form an insoluble precipitate with the chromosomal DNA. The aggregate of SDS with bacterial genomic DNA can be easily separated from the soluble plasmid DNA. In contrast, in the plant system, no precipitation is needed prior to the binding step, because the nonionic non-charged detergent used in the methods and kits described herein does not compete with the DNA for binding sites, since the genomic DNA is the desired binding species for the DEAE groups on the beads. Therefore, the nonionic detergent does not need to be removed, whereas SDS and other anionic detergents are negatively charged

and do compete with DNA for anionic binding sites and therefore would need to be removed.

Example 1: Purification of Plant Genomic DNA.

The following steps describe extraction and purification of DNA from plants.

1. Harvest approximately 0.5 grams fresh plant tissue. Rinse tissue with deionized (d.I). water to remove adhering debris and blot dry.
2. Freeze tissue with liquid nitrogen and grind to a fine powder in a mortar and pestle. Transfer to a 30 mL conical tube. Alternatively, fresh tissue may be mechanically homogenized in cold lysis buffer at 4°C.
3. Add 5-20 ml of lysis buffer/gram of starting material (0.5 to 0.01 M Tris-HCl, pH 7.0 - 8.0, 0.001 - 0.5 M EDTA, pH 7.0 - 8.0, 0.05 - 0.4 M NaCl and 50 - 500 µg/ml proteinase K) to the frozen powder and mix well.
4. Add N-lauroylsarcosine at a final concentration of between 0.1% and 10%. If desired, 25 - 200 µg of RNase A can be added at this point. Alternatively, the final resuspended pellet can be treated with RNase. Incubate 30 minutes to 1 hour at 50-60°C.
5. Centrifuge lysate 10 min at greater than 5000x g at 4°C to pellet debris. Remove the supernatant to a fresh tube and filter, if necessary, to remove undigested debris.
6. Add from 1 - 30 mL of magnetic beads (40 % v/v suspension) to supernatant. Gently mix the bead-supernatant suspension by inversion or mechanical mixer at room temperature for at least 1 minute.
7. Immobilize the magnetic beads in the magnetic separator and remove supernatant.
8. Add between 5 and 20 ml of wash buffer, cap and remove the tube from the stand and gently mix for 5-10 minutes at room temperature.
9. Immobilize the magnetic beads in the magnetic separator and remove supernatant. Repeat wash, if supernatant is substantially pigmented.

10. Add between 5 and 10 ml of elution buffer (0.7 - 1.5 M NaCl, 0.5 to 0.01 M Tris-HCl, pH 7.0 - 8.0, 0.001-0.5 M EDTA, pH 7.0 - 8.0) to the magnetic beads and mix for 5 - 10 minutes at room temperature.

11. Immobilize the magnetic beads in the magnetic separator and transfer supernatant to a fresh tube.

12. Add 1.0 to 2.5 volumes of cold isopropanol. Mix well and incubate at -20°C for at least 10 min.

13. Collect DNA by centrifugation at 10,000 - 20,000 x g for at least 15 min at 4°C. Wash pellet with 70% cold ethanol and re-centrifuge at 10,000 - 20,000 x g for at least 2 min at 4°C. Allow the pellet to air-dry at room temperature and then dissolve in desired buffer.

It is understood that the disclosed method and kit is not limited to the particular methodology, protocols, and reagents described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

Table I: Representative Detergents which can be used in the Plant DNA Extraction Method (available from Sigma Chemical Co)

Anionic Detergents

Aerosol 22
Aerosol®-OT
Salts of:
Alginic Acid
Caprylic Acid
Cholic Acid
1-Decanesulfonic Acid
Dehydrocholic Acid
Deoxycholic Acid
Dioctyl Sulfosuccinate
1-Dodecanesulfonic Acid
Glycocholic Acid
Glycodeoxycholic Acid
1-Heptanesulfonic Acid
1-Hexanesulfonic Acid
N-Lauroylsarcosine
Lauryl Sulfate (Dodecyl sulfate)
1-Nonanesulfonic Acid
1-Octanesulfonic Acid
1-Pentanesulfonic Acid
Taurocholic Acid
Taurodeoxycholic Acid
Niaproof (formerly Tergitol)

Cationic Detergents

Alkyltrimethylammonium Bromides
Benzalkonium Chloride
Benzothonium Chloride
Benzyldimethyldodecylammonium Bromide
Benzyldimethylhexadecylammonium Chloride
Benzyldimethyletetradecylammonium Chloride
Cetyldimethylethylammonium Bromide
Cetylpyridinium
Decamethonium Bromide
Dimethyldioctadecylammonium Bromide
Methylbenzethonium Chloride
Methyltrictylammonium Chloride
N, N',N'-Polyoxyethylene(10)-N-tallow-1, 3-diaminopropane

Zwitterionic Detergents

CHAPS
CHAPSO
N-Decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate
N-Dodecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate
N-Hexadecyl-N, N-dimethyl-3-amonio-1-propanesulfonate
N-Octadecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate
N-Octyl-N, N-dimethyl-3-ammonio-1-propanesulfonate
Phosphatidylcholine
N-tetradecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate

Table I continued.

Nonionic Detergents

BIGCHAP
Decanoyl-N-methylglucamide
n-Decyl -D-Glucopyranoside
n-Decyl -D-Glucopyranoside
n-Decyl -D-Maltopyranoside
Deoxy-BIGCHAP
n-Dodecyl -D-Glucopyranoside
n-Dodecyl -D-Maltoside
n-Dodecyl -D-Maltoside
Heptanoyl-N-methylglucamide
n-Heptyl -D-Glucopyranoside
N-Heptyl -Thioglucopyranoside
n-Hexyl -D Glucopyranoside
Igepal CA-630
1-Monooleoyl-rac-glycerol
Nonanoyl-N-methylglucamide
n-Nonyl -D-Glucopyranoside
n-Nonyl -D-Glucopyranoside
Octanoyl-N-methylglucamide
n-Octyl -D-Glucopyranoside
n-Octyl -D-Glucopyranoside
Octyl -D-Thiogalactopyranoside
Polyoxyethylene Esters
Polyoxyethylene Ethers
Polyoxyethylenesorbitan Esters
Sorbitan Esters
Tergitol
n-Tetradecyl -D-Maltoside
Tritons
Tyloxapol
n-Undecyl -D-Glucopyranoside

We claim:

1. A method for extracting DNA from plant material comprising

(a) lysing the cells in the plant material,

(b) removing the plant cell material debris,

(c) binding the DNA to positively charged groups which are immobilized or insoluble,

(d) separating the bound DNA from the remaining cellular material, and

(e) separating the bound DNA from the positively charged groups.

2. The method of claim 1 wherein a nonionic detergent is used to lyse the cells.

3. The method of claim 1 wherein the positively charged groups are DEAE.

4. The method of claim 1 wherein the positively charged groups are on magnetic beads.

5. The method of claim 1 wherein the plant cell debris is removed by centrifugation.

6. The method of claim 1 wherein the bound DNA is separated by increasing salt ion concentration.

7. The method of claim 1 further comprising adding RNA to digest the RNA in the plant material or purified DNA.

8. A kit for use in a method for extracting DNA from plant material comprising

(a) a solution for lysing the cells in the plant material,

(b) positively charged groups which are immobilized or insoluble, and

(c) a salt solution for separating the bound DNA from the positively charged groups.

9. The kit of claim 8 wherein the solution to lyse the cells is a non-ionic solution.

10. The kit of claim 8 wherein the positively charged groups are DEAE immobilized on a chromatrographic substrate or magnetic beads.

11. The kit of claim 8 comprising:

(a) lysis buffer comprising 0.5 to 0.01 M Tris-HCl, pH 7.0 - 8.0, 0.001 - 0.5 M EDTA, pH 7.0 - 8.0, 0.05 - 0.4 M NaCl and 50 - 500 μg/ml proteinase K);

(b) N-lauroylsarcosine;

(c) magnetic beads having DEAE groups on the surface thereof; and

(d) elution buffer comprising 0.7 - 1.5 M NaCl, 0.5 to 0.01 M Tris-HCl, pH 7.0 - 8.0, 0.001-0.5 M EDTA, pH 7.0 - 8.0.

12. The kit of claim 8 further comprising RNA.

13. The kit of claim 11 further comprising RNA.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 00/10834

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X Y	WO 96 18731 A (DZIEGLEWSKA HANNA EVA ;DYNAL AS (NO); LARSEN FRANK (NO); DEGGERDAL) 20 June 1996 (1996-06-20) page 5, line 30 -page 6, line 11 page 7, line 2 - line 15 page 7, line 28 -page 8, line 1 page 11, line 3 - line 12 --- -/--	1,2,4-9, 12 1-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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